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# High Proportion of New Mutations and Possible Anticipation in Brazilian Facioscapulohumeral Muscular Dystrophy Families

Mayana Zatz,<sup>1</sup> Suely K. Marie,<sup>2</sup> Maria Rita Passos-Bueno,<sup>1</sup> Mariz Vainzof,<sup>1</sup> Simone Camplotto,<sup>1</sup> Antonia Cerqueira,<sup>1</sup> Cisca Wijmenga,<sup>3</sup> George Padberg,<sup>4</sup> and Rune Frants<sup>3</sup>

<sup>1</sup>Centro de Miopatias, Departamento de Biologia, Instituto de Biociências and <sup>2</sup>Departamento de Neurologia, Faculdade de Medicina, Universidade de São Paulo, São Paulo; <sup>3</sup>Department of Human Genetics, Leiden University, Leiden; and <sup>4</sup>Department of Neurology, University Hospital Nijmegen

## Summary

A gene responsible for facioscapulohumeral muscular dystrophy (FSHD) has been localized at 4q35. Subsequently, it was found that probe p13E-11 detects a polymorphic *Eco*RI fragment, usually >28 kb, in normal individuals, whereas in sporadic and familial FSHD cases, an *Eco*RI fragment, usually <28 kb, was found. Although these findings have been amply confirmed, several aspects are as yet either controversial or unsolved. In the present investigation, 34 Brazilian FSHD families were studied at the clinical and the molecular level for the following purposes: to assess the frequency of new mutations and their effect on estimates of biological fitness, to characterize FSHD-associated *Eco*RI fragments detected with probe p13E-11 in familial—as compared with isolated—FSHD cases, and to assess whether anticipation occurs in multigenerational families. Results from our study suggest that new mutations are apparently frequent for FSHD and may account for at least one-third of the cases, that somatic mosaicism may not be rare, and that biological fitness appeared to be reduced in FSHD, ranging from 0.6 to 0.82 by different estimates, with no difference in sexes. Interestingly, the size of the new *Eco*RI fragment is apparently smaller in more severely affected isolated patients. Moreover, the age at onset of clinical signs, as well as the age at ascertainment, in patients from multigenerational families suggests that anticipation occurs for FSHD in the majority of the families.

## Introduction

Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal dominant neuromuscular condition that charac-

teristically affects the facial and shoulder girdle muscles, although there is a marked intra- and interfamilial variability in expression. The penetrance of the gene has been estimated at 95% for patients aged  $\geq 20$  years (Lunt et al. 1989), and life expectancy is almost normal (Padberg 1982; Munsat 1986).

The gene responsible for FSHD was localized to 4q35 by linkage with polymorphic markers (Wijmenga et al. 1991, 1992b). These findings were confirmed by several other groups worldwide (Sarfarazi et al. 1992; Passos-Bueno et al. 1993), although genetic heterogeneity seems to occur in some families (Gilbert et al. 1993; Iqbal et al. 1992; Bakker et al., in press). Subsequently, it was observed that in normal individuals, the probe p13E-11 detects a polymorphic *Eco*RI fragment that is usually >28 kb, whereas in FSHD families a specific shorter fragment, usually between 14 and 28 kb, was found to cosegregate with FSHD (Wijmenga et al. 1992a; Passos-Bueno et al. 1993). Characterization of the polymorphic fragments demonstrates that they consist of multiple copies of a 3.3-kb tandem repeat and that in FSHD patients an integral number of these tandem repeats are deleted (Wijmenga et al. 1993).

Recent analysis of FSHD families raised several clinical, genetic, and molecular questions. If the frequency of a condition does not vary from generation to generation, the number of cases arising as a result of new mutations should be equal to the number that is eliminated because of reduced fitness. Since FSHD is apparently compatible with normal fertility, Morton and Chung (1959) predicted a low mutation frequency in this disease.

However, recent DNA studies suggest that new mutations may not be rare for FSHD (Wijmenga et al. 1992a; Griggs et al. 1993). Although it is well established that deletions lead to FSHD, it is not known whether there is a correlation between the size of the *Eco*RI fragment detected with p13E-11 and the severity of the phenotype or whether it differs in inherited cases as compared with cases that have arisen through new mutations.

The occurrence of anticipation, that is, an earlier onset with increase in the severity of clinical symptoms in subsequent generations, has been investigated recently for several conditions with variable expression (Aslanidis et al.

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Address for correspondence and reprints: Dr. Mayana Zatz, Centro de Miopatias, Departamento de Biologia, Instituto de Biociências, Universidade de São Paulo, Rua do Matão 277, CEP 05508-900, São Paulo, Brazil.

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1992; Buxton et al. 1992; Harley et al. 1992; MacDonald et al. 1993; Orr et al. 1993). This phenomenon has apparently not been reported in the case of FSHD. Here we report on these intriguing questions based on the results of 34 FSHD families that were clinically, neurologically, and genetically investigated.

## Patients and Methods

### Patients

Patients and families were studied at the Centro de Miopatias, Universidade de São Paulo. Patients (at least one from each family) as well as at-risk relatives (parents, sibs, and offspring of affected patients) were clinically and neurologically examined in our center. The diagnosis was based on clinical and family history, electromyography, and muscle biopsy, following the four main criteria defined by the International FSHD Consortium (Padberg et al. 1991). Of the 34 families examined, 19 were multigenerational, while in 15 the proband was the first case in the family. Patients' relatives were classified as nonaffected if they had no clinical signs of FSHD and were >20 years of age and as "at risk" if they were <20 years of age.

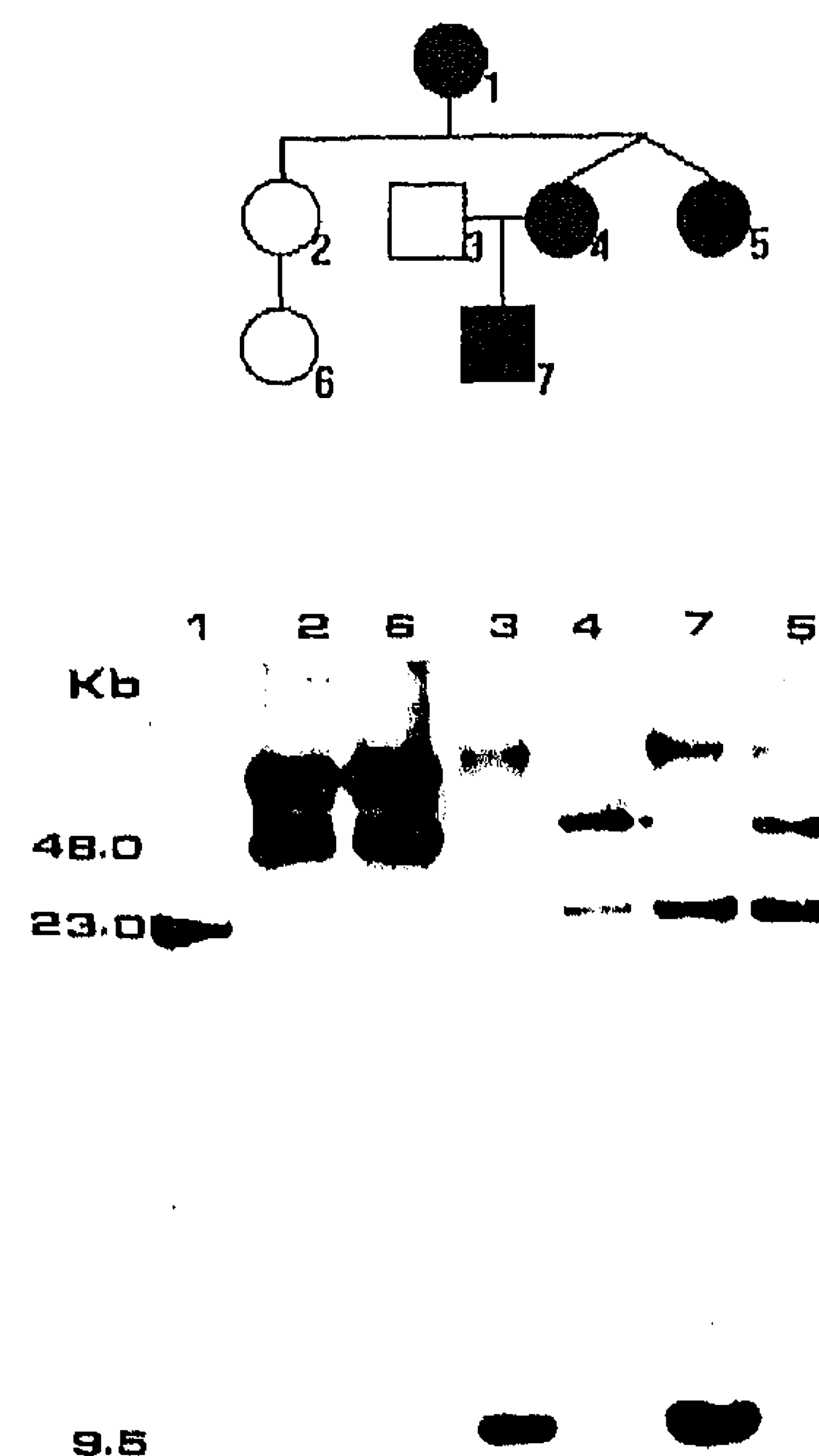
For adults, the age at onset was estimated as the age when the patient first noticed any evidence of muscular weakness and, for affected children, when the patients' parents first observed any sign of FSHD. Whenever possible, this information was confirmed by the referring physician and by other relatives. Patients in whom the neurological examination showed some pathological signs but who were still asymptomatic were not included in the analysis. Age at ascertainment was estimated as the first time the family visited our center and the patient displayed clinically defined symptoms.

### Molecular Genetic Analysis Technology

DNA was extracted from whole blood according to the method of Miller et al. (1988) and was digested with *Eco*RI and electrophoresed in a 0.5% agarose gel, as reported previously (Passos-Bueno et al. 1990b). Analysis of the polymorphism detected by probe p13E-11 was performed according to the method of Wijmenga et al. (1992a) and Passos-Bueno et al. (1993).

DNA was analyzed for a total of 155 individuals (from the 34 genealogies) who had been clinically and neurologically examined: 77 were clinically affected, 24 were at risk, and 54 were nonaffected. DNA analysis was repeated at least twice in all the isolated and almost all the familial cases and was included in the analysis only when confirmed on independent blots.

Three families in which clinical findings were compatible with FSHD were excluded for the following reasons: In one two-generation family, no small *Eco*RI fragment was found in the affected patients. Analysis of the family with the VNTR marker D4S163, which is closely linked to the



**Figure 1** Southern blot analyses of *Eco*RI-digested DNA hybridized to probe p13E-11 in affected patients from a three-generation family. All patients show the same-size 23-kb fragment, which is considered as the linked FSHD band. The other bands (>48 kb and 9.5 kb) are not specific to chromosome 4 (see text).

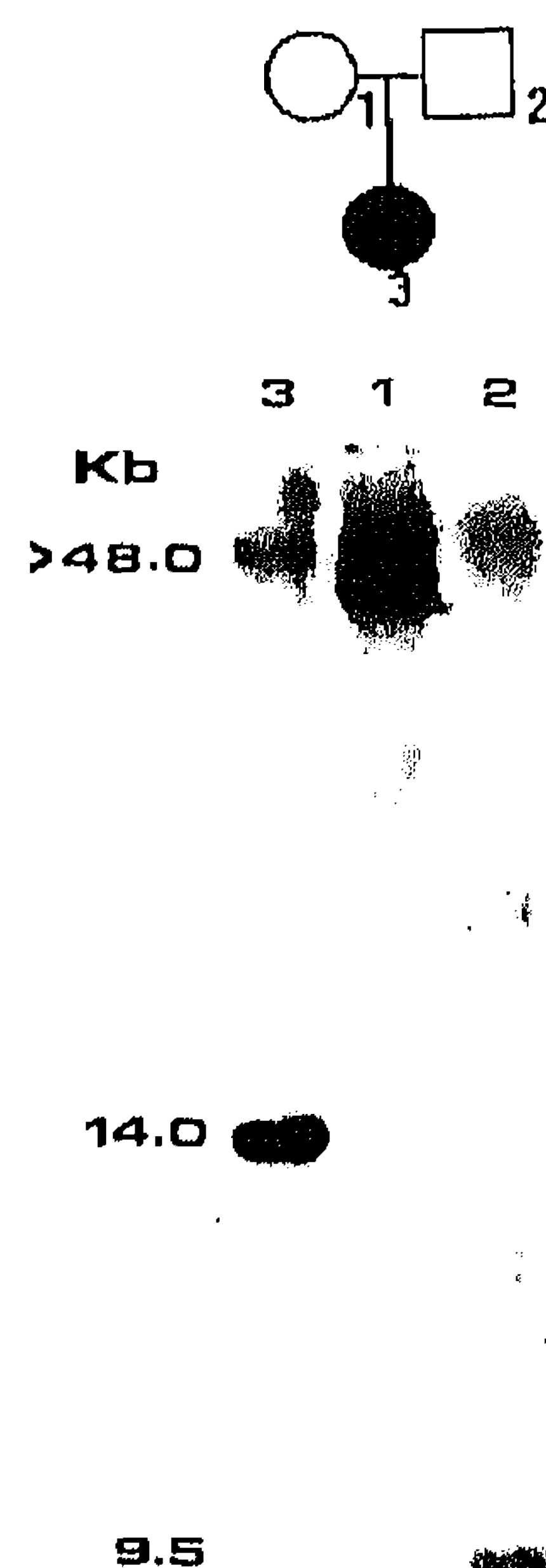
FSHD locus (Sarfarazi et al. 1992), excluded this locus as responsible for the FSHD phenotype in this particular family (lod score =  $-2.8$  at  $\theta = .01$ ). A second two-generation family was excluded because a 30-kb *Eco*RI fragment detected in the first DNA analysis was not seen in subsequent blots; in the third family, in which the proband was an isolated case, three bands were seen, and it was not possible to establish which of the *Eco*RI fragments was associated with FSHD.

Thirty-one FSHD genealogies have, therefore, been included in the present study. Among them, 17 were familial cases, and in 14 the proband was the first case in the family. Students' *t*-tests (paired and unpaired) and  $\chi^2$  analysis were used for statistical analysis, as detailed in Results.

## Results

### Analysis of the *Eco*RI Fragment

Although the size of the p13E-11 *Eco*RI fragment varied from one family to another, in all familial cases the same-sized fragment was segregating among affected patients within each individual family (fig. 1). An *Eco*RI fragment of <30 kb was observed in every case among the isolated cases (fig. 2). The size of the *Eco*RI fragment was, on average, larger in familial cases ( $22 \text{ kb} \pm 4.8$ ,  $n = 17$ ) than in isolated cases ( $18 \text{ kb} \pm 5.5$ ,  $n = 14$ ), but statistical analysis showed only a borderline level of significance ( $t = 2.09$ ;  $P = .05$ ).



**Figure 2** Southern blot analyses of *Eco*RI-digested DNA hybridized to probe p13E-11 in an isolated case. The novel *Eco*RI fragment present in the patient is not seen in the unaffected parents. The other bands (>48 kb and 9.5 kb) are not specific to chromosome 4 (see text).

To estimate the proportion of cases due to new mutations, two parameters were taken into consideration: (a) the number of normal sibs of the index case and (b) the analysis of the novel *Eco*RI fragment in the proband and in the parents. For this analysis 3 of 14 families were excluded; in 2 families, the proband's parents were not available, making it impossible to determine whether a new mutation had occurred, and in the third case, where the father could not be tested, the proband's mother and three apparently normal sibs who were tested did not carry the novel *Eco*RI fragment present in the proband. However, three other sibs (two of them <20 years of age) could not be tested.

In 7 of the 11 remaining families, both parents were tested, and none of them have the small *Eco*RI fragment observed in the index case. In the other four families, the parents were deceased or not available but were inferred (by their physician or relatives) to be unaffected. In these families, the probability of new mutation events is high ( $P > .98$ ), because the probands have 6 to 10 normal sibs, all >20 years of age. Therefore, when the number of unaffected individuals in each family is taken into account, together with the results of DNA, it was estimated that new mutations are highly probable in 11 of the 14 isolated cases.

One particular family caught our attention. The proband, a 30-year-old female patient, showed a typical FSHD phenotype with considerable weakness of her face and upper-limb musculature. She had three unaffected sibs, and clinical examination did not reveal any weakness in her

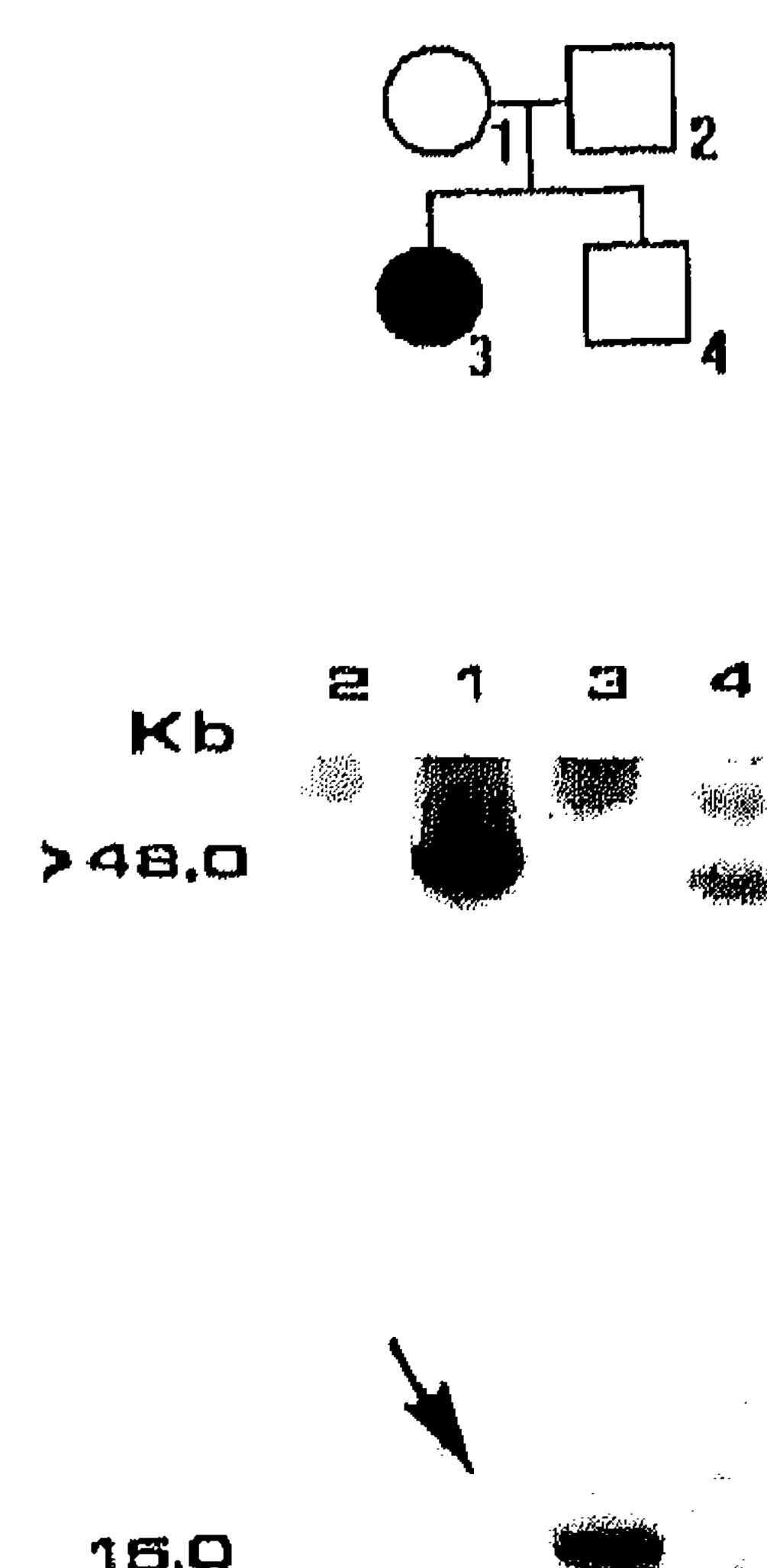
parents, suggesting that she carried a new mutation. DNA analysis showed a strong 15-kb *Eco*RI band in this proband. Interestingly, a band of the same size—but extremely faint—was detected in her unaffected mother, in independent blots, suggesting maternal mosaicism (fig. 3).

### Analysis of Anticipation

In order to assess whether anticipation occurs in multigenerational families, the age at onset, as well as age at ascertainment (table 1), was estimated for the affected offspring and was compared with those of their affected parents, with (a) all subjects (unpaired *t*-test) and (b) pairs ( $n = 28$ ) of affected parent/child (paired *t*-test) taken into account. On average, the onset of clinical signs (as well as the ascertainment) occurred significantly earlier in the affected offspring as compared with their parents, considering all subjects or pairs of affected parent/child. In multigenerational families, the difference in the mean age at onset, between parents (32.3 years) and offspring (15.0 years), was 17.3 years, while the difference in the mean age at ascertainment, between parents (51.2 years) and offspring (26.1 years), was 25.1 years.

In order to verify whether such differences could be due to a bias of ascertainment, since more severely affected probands are more likely to be ascertained, statistical analysis was repeated excluding the index case from each family. As seen in table 1, the statistically significant differences were confirmed, both for age at onset and for age at ascertainment, for affected parents when compared with their affected offspring.

In order to assess whether the gender of the transmitting parent could influence the age at onset in affected off-



**Figure 3** Maternal mosaicism showing that the same strong 15-kb *Eco*RI fragment seen in the affected proband is present as a faint band (arrow) in her unaffected mother. The same results were obtained in three different blots in which the DNA concentrations were comparable for all subjects in this family (data not shown).



**Table 1****Mean Ages at Onset and at Ascertainment in FSHD Families**

Group (N)	Age at Onset <sup>a</sup> (years)	Age at Ascertainment <sup>b</sup> (years)
A. Familial cases: total sample:		
1. Parents (17) .....	32.3 ± 19.8	51.2 ± 13.7
2. Offspring (37) .....	15.0 ± 5.8	26.1 ± 9.8
B. Familial cases: without probands:		
1. Parents (14) .....	34.4 ± 21.1	54.1 ± 13.0
2. Offspring (24) .....	15.4 ± 5.6	26.4 ± 10.6
3. Probands (17) .....	15.8 ± 7.1	27.6 ± 9.0
C. Isolated cases (14) .....	14.5 ± 9.5	27.2 ± 11.0

<sup>a</sup> By unpaired *t*-test, differences between groups A.1 and A.2 ( $t = 4.9$ ;  $P < .01$ ) and between groups B.1 and B.2 ( $t = 4.2$ ;  $P < .01$ ) are highly significant whereas differences between groups B.2 and B.3 ( $t = -.19$ ;  $P > .05$ ) and between groups B.3 and C ( $t = -.45$ ;  $P > .05$ ) are not statistically significant; by paired *t*-test, differences between A.1 and A.2 are highly significant ( $t = 8.9$ ;  $P < .01$ ).

<sup>b</sup> By unpaired *t*-test, differences between groups A.1 and A.2 ( $t = 7.8$ ;  $P < .01$ ) and between groups B.1 and B.2 ( $t = 7.8$ ;  $P < .01$ ) are highly significant whereas differences between groups B.2 and B.3 ( $t = -.36$ ;  $P > .05$ ) and between groups B.3 and C ( $t = -.12$ ;  $P > .05$ ) are not statistically significant; by paired *t*-test, differences between A.1 and A.2 are highly significant ( $t = 18.2$ ;  $P < .01$ ).

spring, patients born from affected mothers were compared with those born from affected fathers. No statistically significant differences were observed between the two groups: the age at onset was  $14.5 \pm 6.31$  years ( $n = 20$ ) for group 1 and  $15.7 \pm 5.92$  years ( $n = 17$ ) for group 2 ( $t = .6$ ;  $P > .05$ ).

### Fitness Estimate

Biological fitness was estimated by means of two approaches. The first involves calculating the total number of children born from all affected patients, as compared with the total number of children born from their normal unaffected sibs of comparable age (all were  $>20$  years of age). In this analysis, 29 families were included. The average number of children of FSHD patients was 1.86 (104/56) as compared with 2.26 (276/122) for their normal sibs, which led to a fitness of 0.82 ( $1.86/2.26$ ), with no difference between sexes (0.81 for affected females and 0.84 for affected males).

The second approach is a simple method proposed by Tanaka (1974), which is particularly valuable for autosomal dominant disorders (Emery 1976), where  $f = A_p/A_o$  ( $A_p$  = frequency of the trait among parents of index cases; and  $A_o$  = frequency of the trait among offspring of index cases). In this analysis, 14 three-generation families in which the probands' descendants were  $>20$  years of age were included. It was found that  $A_p = .32$  (9 of 28 probandus' parents were affected) and  $A_o = .53$  (27 of 51 probandus'

descendants were affected), which led to a fitness estimate of 0.6 ( $.32/.53$ ).

## Discussion

### Proportion of New Mutations and Somatic Mosaicism

Somatic and/or germinal mosaicism has been reported for several genetic diseases (Maddalena et al. 1987; Bakker et al. 1989; Brocker-Vriends et al. 1990; Passos-Bueno et al. 1990a). For Duchenne muscular dystrophy (DMD), it has been shown that the frequency of cases resulting from germinal mosaicism depends on the site of the deletion in the dystrophin gene (Passos-Bueno et al. 1992).

Somatic mosaicism has been previously reported for FSHD (Griggs et al. 1993; Weiffenbach et al. 1993), but its frequency is still unknown. It would be of interest to assess whether the mosaicism detected in peripheral blood in the probandus' mothers from the present study is also present in other tissues, in particular in skeletal muscle. However, such an analysis requires a muscle biopsy, which is difficult to obtain in the present case. The fact that the transmitting mother is clinically asymptomatic does not allow any speculation about the proportion of muscular cells potentially carrying the defective gene, since subclinical cases are known for individuals from proved chromosome 4q-linked families.

The present observation of one mosaic case of seven in which both parents could be studied suggests that it might not be rare for FSHD, but further studies are required to confirm this hypothesis. Such findings, in addition to being indicative of somatic origin for new mutations, are important for recurrence-risk calculations, as already shown for DMD (Passos-Bueno et al. 1992).

The large proportion of isolated cases (14/31, or 45%) in the total sample called our attention, because the proportion of cases resulting from new mutations had previously been assumed to be very low (Morton and Chung 1959; Thompson 1986). Our results, however, suggest that new mutations are not rare and may account for at least one-third of the cases. This observation supports the original report by Wijmenga et al. (1992a) who detected de novo rearrangements in 5 of 15 FSHD families, in which 6 were isolated cases, although this sample was the first to be studied and might not be representative of the Dutch population. Recently, Griggs et al. (1993) reported the occurrence of new mutations in eight sporadic FSHD patients, on the basis of clinical findings and the analysis of the novel *EcoRI* fragment in the probands and in their parents.

Finding a relatively high proportion of new mutations is not compatible with previous studies, which reported high fertility (Morton and Chung 1959) and apparently normal fitness for FSHD (Eggers et al. 1993). According to Emery (1976), however, a reliable estimate of biological fitness is difficult to determine, because of insufficient data. In some



of the earlier studies, the clinical overlap of limb-girdle cases with cases of scapulohumeral onset led some investigators to pool both types into a single group. In the case of FSHD, previous estimates of normal fitness, including our own earlier study (Eggers et al. 1993), were based on multigenerational families only. However, when we reanalyzed the reproductive performance of affected patients, including the isolated cases, according to the method of Tanaka (1974), we found a reduction in biological fitness. This method is based on the principle that if selection against a specific disorder is sufficiently strong, the frequency of affected individuals among parents of index cases will be lower than that among the offspring of index cases and that this reduction is proportional to the intensity of selection. The advantage of this method is that it takes into account the cases that have arisen from new mutations and not the total number of descendants. Interestingly enough, according to Morton and Chung (1959), although FSHD is compatible with high fertility, they also estimated a reduced fitness of  $\sim 0.736$  for this condition.

In view of the unexpectedly significant proportion of isolated cases, it would be important to have fitness reassessed in FSHD patients from other populations, through different methods, with an effort being made, whenever possible, to see all patients within a given population. In any case, the observation that new mutations are not rare for FSHD has important implications for clinical diagnosis and genetic counseling, in particular of isolated cases.

#### *Analysis of the EcoRI Fragments*

The present report supports previous studies showing that in 31 Brazilian families, the clinical picture of FSHD is caused by a gene located at 4q35, closely linked with *EcoRI* fragments ranging from 10.5 to 33 kb. Probe p13E-11 detects polymorphic restriction fragments from several loci, some of which are difficult to resolve by conventional agarose gel electrophoresis (Wijmenga et al. 1994). Only a number of these loci have been chromosomally mapped. A 9.5-kb fragment appears only in males and consequently maps to the Y chromosome. In the largest size range, two polymorphic loci can be visualized by pulsed-field gel electrophoresis. Recently we were able to map the second locus to the long arm of chromosome 10, in the region q35 (Bakker et al., in press). Interestingly, the average size of the 10q fragments is smaller than the average size of the 4q fragments (Wijmenga et al. 1994). As FSHD is usually associated with small (<30 kb) *EcoRI* fragments, this second locus may hamper the interpretation of conventional Southern blots and forms a serious caveat for FSHD diagnosis in small families. For diagnostic purposes, haplotyping with 4q and 10q markers might help.

#### *Recombinant/Position Effect*

The observation of both the new "small" *EcoRI* fragments in new FSHD patients and cosegregation in families

suggested a causal relationship between the rearrangement and the appearance of FSHD (Wijmenga et al. 1990, 1992a). The rearrangement was shown to be due to deletion of integral copies of the 3.3-kb repeat unit (van Deutekom et al., in press). The most straightforward explanation is that the FSHD gene is structurally affected through the deletion. There are, however, several observations making this model less obvious, suggesting a functional rather than a structural dysfunction of the FSHD gene. Several cases of apparent affected recombinants in FSHD families with no small fragment have been reported (Upadhyaya et al. 1993; Weiffenbach et al. 1993). Although several of these recombinants could reflect diagnostic misclassifications, a few have withstood severe scrutiny. These recombinants can be accounted for by intragenic and interlocus recombination events or by other, as yet unidentified, mechanisms (Wijmenga et al. 1993; Winokur et al. 1994).

Analysis of the p13E-11 locus has revealed it to be very close to the telomere and to contain sequences often encountered in heterochromatic regions (Hewitt et al. 1994; Winokur et al. 1994). These observations are in line with a position-variegation effect involved in the etiology of FSHD and might suggest that the FSHD gene is located either upstream or downstream of the *EcoRI* fragment. So far, no signs of transcriptional activity have been found in the telomeric part. As the search for a transcript within the repeat units has been unsuccessful, the position effect deserves attention. Consequently, the gene search should be directed upstream.

#### *Clinical Variability*

The great variability in the severity of the phenotype, a well-known characteristic of FSHD, was also confirmed in our study, with the age at onset of clinical signs ranging from 2 to 50 years. In two unrelated multigenerational families with multiple affected patients, the gene was nonpenetrant, since parents of affected patients (a female aged 46 years and a male aged 79 years), who were the proved transmitters of the FSHD gene (through pedigree analysis, since both had clinically affected sibs), were asymptomatic on clinical examination. In both families, the same specific *EcoRI* fragment found in affected patients was present in the asymptomatic carriers of the FSHD gene. The occurrence of a nonpenetrant case of FSHD in a 61-year-old female was also recently reported by Griggs et al. (1993).

The difference in the average size of the fragment in isolated cases, as compared with familial cases, although smaller in the former group, showed a borderline level of significance. However, when the size of the fragment was analyzed in relation to the severity of the phenotype among isolated cases, we observed that among the more severely affected patients (onset before the age of 10 years) the average size of their *EcoRI* fragment ( $12.9 \text{ kb} \pm 2.0$ ,  $n$



= 4) was significantly smaller than that among patients with later onset ( $20.0 \pm 5.7$  kb,  $n = 10$ ,  $P < .01$ ).

These differences were surprising, since within each multigenerational family, the same-size *EcoRI* fragment is segregating in patients with different degrees of clinical severity. Although the number of patients is small, and this result should be confirmed in a larger sample, it suggests that smaller fragments may be associated with a more severe phenotype.

### Analysis of Anticipation

Anticipation is a phenomenon that has been extensively described for myotonic dystrophy (DM) and other conditions, such as Huntington disease (HD) and spinocerebellar ataxia (MacDonald et al. 1993; Orr et al. 1993), that have both an increase in severity and earlier age at onset in successive generations. In the present sample, with the exception of one family in which the mother (aged 52 years, onset at the age of 18 years) was more severely affected than her son (aged 32 years, onset at the age of 24 years), all showed, on average, an earlier onset in the younger than in the older generation. As seen in table 1, such a difference was even greater when the analysis was repeated excluding the proband of each family.

Our present observation of a mean age at onset 17–19 years earlier, on average, in affected offspring, as compared with their parents in multigenerational families (after correction for a possible bias of ascertainment toward more severely affected probands), has apparently not been previously reported for FSHD. A common criticism of this kind of study is that data on age at onset of clinical symptoms in earlier generations are not always reliable. The age at onset in parents, however, as compared with their affected children, was considered by Penrose (1948) for the analysis of anticipation in DM. In the present study, such information was confirmed in most of the cases through clinical examinations and follow-up, in some cases for >20 years in our own center. Some affected parents who reported later onset have such a mild phenotype that they are currently less handicapped than their affected offspring who are, on average, 20–30 years younger. In addition, in the present analysis, the results observed on the basis of age at onset are reinforced by those obtained on the basis of age at ascertainment.

Although the present study supports the hypothesis of anticipation for FSHD in multigenerational families, this disease differs from other autosomal dominant conditions with anticipation such as DM and HD, in three aspects: (a) the lack of difference in the severity of the phenotype according to the gender of the transmitting parent; (b) the high proportion of cases arising from new mutations—as opposed to DM and HD, in which new mutations are apparently very rare; and (c) the reduced biological fitness.

Interestingly, no statistically significant differences were observed for both the age at onset and the age at ascertain-

ment, between the probands in familial cases, as compared with isolated cases (table 1). This observation supports prior knowledge that more severely affected cases are more likely to come to medical attention earlier and that isolated cases with visible symptoms seek medical help at the same age, on average, as do familial cases. We are aware, however, that we are probably not ascertaining isolated patients with a mild phenotype. Results from the present study are yet to be confirmed in a larger sample of FSHD families from different populations. Revealing the molecular defect in FSHD is of great importance to elucidate the relationship between genotype and phenotype.

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